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A SYSTEM FOR PREPARATION, DETECTION AND IDENTIFICATION OF A PRECIPITINOGEN (η) OF STRAINS OF *MYCOBACTERIUM TUBERCULOSIS*, THAT IS NOT DETECTABLE IN STRAINS OF *M. BOVIS*[1,2]

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SUMMARY A precipitinogen (designated η: eta), which was undetectable in BCG and the Ravenal strain of *Mycobacterium bovis*, was detected in sonic extracts of the H37Rv and H37Ra strains of *M. tuberculosis* by agar gel immunodiffusion. Since precipitinogen η was very labile in cell extracts and crude preparations, reproducible methods for preparing and detecting the precipitinogen η were investigated. Two conditions were found necessary for a stable preparation: (1) deferration of all materials to which η precipitinogen was exposed throughout procedures and (2) use of phosphate buffer of over pH 7.5 and over 0.07 *M* for solubilizing η. Two methods were effective and useful for purification of precipintiogen η: (1) Salting-out using deferrated ammonium sulfate (35–40% saturation) and (2) gel permeation chromatography on Sephadex G-200 or Bio-gel P-300 that had been thoroughly washed with chelating agents. The purified η prepared by a combination of these two methods, retained activity on storage in the frozen state for several years or on incubation at 40 C for 30 min. A system was developed for detection and identification of η using stable, purified η as a reference. η Precipitinogen was heat-labile (60 C for 10 min), and behaved as an acidic protein with a molecular weight of 230,000~260,000 daltons.

INTRODUCTION

Taxonomical differentiation of *Mycobacterium tuberculosis* (*M. tbc*) from *M. bovis* by various biochemical methods, including the niacin test (Konno, 1956; Runyon et al, 1959) and reduction of nitrate (Virtanen, 1960; Wayne and Doubek, 1968; Escoto and de Kantor, 1978) was established in the last 10 years. But no biochemical reaction unique to *M. tbc* species

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3 Died on August 29, 1976.

is known (Kubica, 1973; Runyon et al., 1974; Barksdale, 1977). Moreover, no biochemical or antigenic character of *M. tbc*, that is specifically associated with its difference in pathogenicity for various hosts has yet been found, although Boyden and Sorkin pointed out this fact more than 20 years ago (Boyden and Sorkin, 1956). In antigenic analyses, *M. tbc* has generally been considered to share all known antigens with *M. bovis*, although *M. tbc* and *M. bovis* are clearly distinct from other species of mycobacteria (Wilson, 1925; Lind, 1978). From immunochemical analyses, Stanford and Grange (1974) allocated *M. tbc*, *M. bovis*, *M. microti* and *M. africanum* to a single serological species corresponding to the tuberculous group in the taxonomy of mycobacteria. "Species-specific" has been used to describe the antigens shared with the tuberculous group but not with other species of mycobacteria. So-called "specific" antigens of *M. tbc*, defined by precipitinogen or the tuberculin activity reported by Fauser (1969) and Daniel et al. (1976, 1978, 1979), were actually common to *M. bovis* BCG. "Specific" antigens prepared from *M. tbc* were also reported by Seibert and Soto-figueroa (1957), Knicker and laBorde (1964), Stanford and Beck (1968), Stottmeier et al. (1969), Moulton et al. (1972), Bardana et al. (1973), Stanford and Grange (1974), Turcott (1975) and Nassau and Nelstrop (1976) who did not test their relation to BCG antigens. On the other hand, Chaparas (1975) concluded that no antigens unique for *M. tbc* were found from comparative studies on the antigens of 12 species of mycobacteria using a *M. tbc* reference system (Janicki et al., 1971). However, Turcott and Boulanger (1971) demonstrated antigens specific to virulent strains of *M. tbc* and *M. bovis*, and not detectable in respective avirulent strains. These antigens differed from each other suggesting the presence of strain-specific antigens.

For survey and identification of particular antigens in various preparations, two dimensional immunodiffusion in porous matrices (Ouchterlony, 1948; Axelsen, 1973) is the most suitable of the various serological methods available. For this method, the precipitating antigens should be obtained in a soluble and diffusible form, and should be in a sufficiently native form to retain immunogenic activity to induce the antibodies that are indispensable for the method. However, with mycobacterial preparations, very variable antigenic, and therefore antibody, compositions are obtained, as shown, for instance, by the difference between lot 001 and 002 antisera obtained with the same reference antigen preparation of *M. tbc* by Chaparas et al. (1978b). For use in survey of antigenic characteristics, antisera must contain antibodies against particular antigens. Therefore, we tested various methods for preparing antigen solutions from culture filtrates and cells of strains of *M. tbc* obtained under various cultural conditions in various media, and then prepared rabbit antisera against these antigen preparations. Fortunately one of the antisera formed a precipitation band characteristic of the H37Ra extract (Fig. 1) that did not fuse with any bands formed by BCG and Ravenel extracts. We designated this band formed using this particular antiserum, η(eta), according to H in H37Ra of human tubercule bacilli (*M. tbc*) to avoid confusion with overlapping α, β, γ, etc. antigens so far reported (Yoneda and Fukui, 1965; Norlin and Ernevad, 1966).

Our observations that all extracts of *M. tbc* formed the η band against the antiserum described above, but that the η activity disappeared on storage with concomitant aggregation, suggested that η precipitinogen was very labile in crude extracts. These findings raised the following questions: (1) Is a specific antigen(s) of *M. tbc* actually present? (2) If so, is the antigen(s) single or mutiple? Since η activity in crude extracts was lost in a few days, we could not identify a specific band as η even when patterns of the bands in different experiments were similar (Fig. 1).

To confirm these findings, we assumed that there is only one specific antigen that is detectable in *M. tbc* extracts but not in BCG

extracts, and performed the following experiments: (i) We looked for a reproducible system for preparing and detecting η precipitinogen. (ii) We prepared a purified η precipitinogen stable enough to be stored for use as a reference antigen of η precipitinogen. (iii) We confirmed the presence of specific antigen of *M. tbc* using the purified reference antigen. This paper reports these studies.

MATERIALS AND METHODS

1. *Bacterial strains*

Biken substrains of *Mycobacterium tuberculosis* H37Rv and H37Ra strain, Biken substrains of *M. bovis* BCG (from the National Institute of Health, Tokyo), and the Ravenel strain were used. Strain BCG was used as a reference strain of *M. bovis* for the detection of η precipitinogen.

2. *Preparation of antigens*

For preparation of BCG reference antigens or starting materials for purification of η precipitinogen, organisms were grown by the following procedure: Cells (wet weight, about 1.5 g) grown for 2 weeks at 37 C on glycerine-broth potato (Rosenthal, 1937) were transferred to a 500 ml shaking flask containing 100 ml of the medium described by Yoneda et al. (1969). After incubation for 4 days at 220 rpm on a rotatory shaker at 37 C, 10 ml aliquots of the culture were inoculated into 100 ml volumes of medium without Tween 80 and beads, and shaken for 2 weeks under the same conditions as before. Cells were harvested by centrifugation and stored in a deep-freezer. Frozen cells were thawed and suspended in an appropriate buffer in a volume of about three times that of the cells. The cell suspension (100 ml) was sonicated at 20 kc, 60 w (Branson sonifier^{R} cell disruptor 200) three times (each 10 min) with 10 min intervals in a vessel immersed in an ice-water bath to the tip of the sonicator. The dispersion of biohazadous aerosol was prevented by connecting one end of rubber tube to a stainless steel tube inserted into the rubber stopper for the vessel, and the other end to an in-line type filter (47 mm, SM 13606 membrane filter, Sartorius), to release the air pressure in the closed vessel at the beginning of sonication. The sonicate was centrifuged twice at 100,000 $\times g$ at 4 C for 1 h to precipitate the particulate fractions, and then the clear part of the supernatant was filtered through membrane filter (pore size, 0.2 μ) and used as crude antigen preparation (sonic extract).

3. *Membrane filters*

The membrane filters tested for recovery of η precipitinogen on sterilization or ultrafiltration of the bacterial extracts, were as follows: HA 0.45 μ and 0.22 μ made of mixed esters of cellulose, EH 0.5 μ and EG 0.2 μ made of cellulose acetate, BD 0.6 μ made of polyvinyl chloride, NR 1.0 μ of nylon membrane (Duralon) of Millipore filters (Millipore Corporation, Bradgord, Mass.); SM 11306 0.45 μ and SM 11307 0.2 μ made of cellulose nitrate, SM 11106 0.45 μ and SM 11107 0.2 μ made of cellulose acetate and SM 12806 0.45 μ made of polyvinyl chloride of Sartorius membrane filters (Sartorius GmH, Goettingen).

4. *Preparation of antisera*

Rabbits were injected intramuscularly with 10, 20 and 40 mg protein per rabbit of the sonic extract of the H37Rv or H37Ra strain in complete Freund adjuvant (heat-killed and dried cells of homologous strains, respectively, were used), or with 200 μg protein per rabbit of purified η precipitinogen in incomplete Freund adjuvant. Two weeks after primary injections of 5, 10 and 20 mg or 100 μg, two booster injections one week apart were given (2.5, 5 and 10 mg protein of crude preparation or 50 μg protein of purified preparation). Rabbits were bled 1 week after the last injections. Antisera were partially purified after 4-fold dilution with 0.05 *M* Na K phosphate buffered saline, pH 7.8. The superantant obtained by precipitation with 30% of saturation of ammonium sulfate at 0 C overnight and by centrifugation at 12,000 rpm for 20 min, was treated with dSAS at 25 C (as described in section 11). The precipitate was dissolved in a quarter volume of the original antiserum, and dialyzed against buffered saline (0.02 *M* deferrated phosphate buffer, 0.1 *M* of NaCl (99.99% of purity), 0.02 *M* Na acetate and 0.1% Na azide).

5. *Measurement of protein content*

During the purification procedure, the protein contents of fractions precipitated with 5% trichloroacetic acid, were measured by the method of Lowry et al. (1951) as modified by Hartree (1972) or by the micro-biuret reaction (Itzhaki and Gill, 1964) or from the absorbance at 280 nm.

6. *Agar and agarose*

Special noble agar, Purified agar (Difco), Agarose A37 (Nakarai Chemicals, Ltd., Kyoto), Agarose I, II and III (Dojindo Co., Ltd., Kumamoto) and Agarose (Seakem Biochem.) were tested in Ouchterlony's double diffusion method. In the established system, Agarose A-37 were employed for Ouchterony's method and immunoelectrophoresis.

7. *Immunoelectrophoresis and zone electrophoresis*

Twice concentrated electrode buffers (ionic strength, 0.1) were deferrated by the hydroxylapatite method described in Section 10. Crossed or crossed-rocket-line immunoelectrophoresis was carried out on " deferrated " agarose gel of 1.0 mm thickness on 7×10 cm glass plates as described by Krøll (1973) with a slight modification, at room temperature at a constant voltage of 10 V per cm overnight. After the run, the gels were stained with 0.01% nigrosin (Chroma-Gesellschaft Schneid & Co., Stuttgart-Untertuerkheim) in 2% acetic acid and destained in 2% acetic acid.

Zone electrophoresis was performed by the method of Katsura et al. (1957).

8. *Estimation of molecular weight*

Molecular weight was estimated by gel permeation chromatography through Sephadex G-200 and Sephacryl S-300 (Pharmacia Fine Chemicals) by the method of Andrew (1964) using HMW calibration kits for molecular weight determination (Pharmacia Fine Chemicals).

9. *Preparation of " deferrated " phosphate buffer*

" Deferrated " heavy metal-free phosphate buffer was prepared as follows: 0.3 g of $Ca(OH)_2$ powder suspended in 5 ml of distilled water was dissolved by adding 2 ml of H_3PO_4 solution (85%). This calcium phosphate solution was added to 1 liter of 0.6 *M* NaH_2PO_4 and then the pH was adjusted to 7.9 with 30% (W/V) NaOH solution. The mixture was boiled for 15 min in acid-cleaned Corning glassware or a stainless steel vessel, and then filtered while still hot through filter paper (Whatman No. 40, ash-free or Toyo No. 7). The filtrate was cooled and refiltered through a double layer membrane filter (Sartorius, cellulose nitrate; 0.45 μ and 0.2 μ pore size at the top and bottom, respectively). The concentration of buffer in the filtrate was adjusted with distilled water after determining the phosphate concentration by the method of Baginski (1967) or by measuring electro-conductivity. The deferrated 0.5 *M* phosphate buffer, pH 7.7, thus obtained was stored at room temperature as a concentrated stock solution after distribution in 1 liter aliqouts and autoclaving at 120 C for 30 min.

10. *Preparation of deferrated agar*

For stabilization and reproducible production of the η precipitation band, the agar used for immunodiffusion or immunoelectrophoresis was deferrated as follows: For Ouchterlony's double diffusion agar techniques, Agarose A-37 was dissolved at 0.8% in 0.12 *M* NaCl and filtered through a GFF glass fiber filter (Whatman). The filtrate was mixed with Ca acetate (final 2 m*M*), Na acetate (final 10 m*M*) and deferrated Na phosphate buffer (final 20 m*M*), and incubated at 80 C for 30 min with mixing with a magnetic stirrer. Then it was adjusted with distilled water to the same weight as before incubation and filtered by suction through a GFF filter. The deferrated agar was mixed with NaN_3 (final 0.1%) as preservative and EDTA 3Na salt (final 1 m*M*), and 4 ml portions were spread on glass slides (76×36 mm), solidified, and stored at 4 C in a moist chamber. Wells for antigens or antisera were punched out just before use. For immunoelectrophoresis, 2.4% agarose or LTG agarose (Marine Colloids, Inc.) in distilled water was treated in the same way as described above except that 0.5 g of Hydroxyl Apatite (Seikagaku Kogyo, Tokyo) per 100 ml solution was added instead of forming calcium phosphate gel, and the agar gel was stored in tubes. Before use, the agar was diluted to 1.0 or 0.8% with an appropriate buffer containing antigen or antiserum. The deferration of the agar was checked qualitatively with o-phenanthroline and ascorbic acid.

11. *Deferration of saturated ammonium sulfate solution*

To saturated ammonium sulfate solution (about 5 liters) were added 20~30 ml of ammonium solution at just below the boiling point. After incubation for 15 min, the solution was filtered 3 times repeatedly through the same sheet of filter paper (Whatman No. 40). Then the solution was cooled, and adjusted to pH 7.8 with concentrated H_2SO_4 solution and to a specific gravity of 1.245 (dSAS).

12. *Pretreatment of matrices for column chromatography or zone electrophoresis*

All matrices were washed with hot (80 C for chromatography and 55 C for starch) 0.1 *M* phosphate buffer prepared as described above containing 1 m*M* EDTA and 0.2 m*M* ethylenediamine-di-o-hydroxyphenylacetic acid (EDDHA; Dojin Chemical Institute, Kumamoto) under deaerated conditions by stirring with a magnetic stirrer overnight. This treatment was repeated until no colorization of EDDHA was observed. Then each matrix was washed and equilibrated with the buffer required for the subsequent experiment. The materials tested for column chromatography of η precipitinogen under conditions preventing η from inactivation were as follows: Anion exchangers—DEAE-Sephadex (Pharmacia Fine Chemicals), DEAE-, TEAE-, AE-, GE-, ECTEORA-cellulose (Seikagaku Kogyo, Tokyo); cation exchangers—CM-, P-cellulose (Seikagaku Kogyo, Tokyo), SP-Sephadex (Pharmacia Fine Chemicals); gel permeation matrices—CPG (Whatman), Bio-gel P300 (Bio-Rad Laboratories), Phenyl-Sephadex (Pharmacia Fine Chemicals), Hydroxyl Apatite (Seikagaku Kogyo, Tokyo).

13. *Numerical treatment of data*

Data processing and calculations in the variance analyses of factorial analyses of experimental design were performed with a computor (Olivetti P652 system) programmed by its assembler.

RESULTS

1. *Appearance of a precipitation band specific for sonic extracts of M. tuberculosis, but not of M. bovis BCG, on immunodiffusion plates*

Figure 1 shows the immunodiffusion pattern produced by a cell extract ((SE/Ra)) of *M. tbc* H37Ra strain and its homologous antiserum ([A-Ra]) using a BCG cell extract ((SE/BCG)) as a reference antigen for *M. bovis*. Both extracts ((SE/Ra) and (SE/BCG)) formed many overlapping precipitation bands against the antiserum and these fused with each other, demonstrating the presence of many antigens common to both species. However, one precipitation band that clearly extended straightly toward the wells of (SE/BCG) was observed between (SE/Ra) and the antiserum (Fig. 1). Similar results were obtained with strains H37Rv using BCG or Ravenel extracts.

2. *Determination of optimal conditions for preparing η active sonic extracts and for sterilization of extracts*

However, under the conditions employed in the initial stage of this study, band η could not

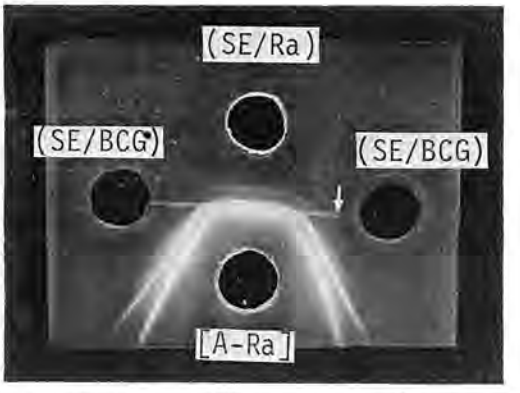

FIGURE 1. Immunodiffusion patterns of sonic extracts of *M. tuberculosis* strain H37Ra ((SE/Ra)) and of *M. bovis* strain BCG ((SE/BCG)) against anti-H37Ra extract serum ([A-Ra]). The arrow indicates the η band. Samples of 10 μl of sonic extracts (5 mg protein per ml) were put in antigen wells.

be detected reproducibly: The η activity was often very unstable in sonic extracts. Moreover, results sometimes depended upon the lots of gel used for immunodiffusion. So, we tried to find out (1) the optimal conditions for preparing sonic extracts and (2) conditions required for reproducible detection of the η band by agar gel diffusion.

Since we observed that sonic extracts of *M. tbc* cells did not form an η band after sterilization by filtration through usual cellulose nitrate membrane filters (Millipore filter HA and GS type, 0.45 μ and 0.22 μ of respective pore size), the 11 kinds of membrane described in the Materials and Methods, were tested. Only Millipore membrane filters made of cellulose acetate permitted η precipitinogen to pass through freely at 4 C when deferrated phosphate buffer was used for preparing the extracts as described below. Sartorius cellulose acetate membranes partially absorbed η precipitinogen. Other membranes retained

TABLE 1. *Summary of results of factorial analysis on constituents of the buffer for preparing η antigen*

Expt. No.	Factors arranged				η-Activity[b] in dialysed sonic extracts
	Buffer (0.05 *M*)	pH	Total salt concentration[a] (*M*)	Metal cation	
1	Tris-Cl	7.0	0.05	—	−
2	Tris-Cl	7.0	0.2	K	−
3	Tris-Cl	8.0	0.05	—	−
4	Tris-Cl	8.0	0.2	Na	+
5	PO_4	7.0	0.05	K	−
6	PO_4	7.0	0.2	Na	−
7	PO_4	8.0	0.05	Na	−
8	PO_4	8.0	0.2	K	++

[a] NaCl or KCl was added at the concentration indicated.
[b] +, visible; ++ clearly visible precipitation band, −, no precipitation band of η.

TABLE 2. *Summary results on factorial analysis of the ingredients of the phosphate buffer for preparing η antigen*

Expt. No.	Factors arranged					η-Activity in dialysed sonic extracts
	pH	Concentration (*M*)	Cation	EDTA[a] (1 m*M*)	β-Mercapte-ethanol (1 m*M*)	
1	7.5	0.1	Na	−	−	+
2	7.5	0.1	K	+	+	−
3	7.5	0.2	Na	+	−	++
4	7.5	0.2	K	−	+	−
5	8.0	0.1	Na	+	+	−
6	8.0	0.1	K	−	−	+
7	8.0	0.2	Na	−	+	−
8	8.0	0.2	K	+	−	++

[a] The cation indicated under *cation* was used to dissolve EDTA-4H.

the η precipitinogen specifically and almost completely.

Then, we made factorial analyses of buffer conditions to determine favorable and unfavorable factors. The effects of the following factors were tested; (i) the kind of buffer salt (Tris-Cl or Na, K-PO_4) (ii) the pH, (iii) the total concentration of salt (iv) the kind of metal cation, (v) chelating agents, (vi) SH-reducing agents and (vii) antiseptics. These factors were set of two levels and arranged in the L8 (2^7) orthogonal array table of experimental design described by Taguchi (1964). Tables 1 and 2 show the results of two representative examples of experiments. Conclusions were deduced by variance analyses (Cochran and Cox, 1957; Taguchi, 1964) (in which 0 was taken as (−), 1 as (+) and 2 as (++)), after all results from the experiments designed by orthogonal arrangements of factors had been collected and rearranged for each factor.

Eight Visking tubes containing 2 ml of H37Ra sonic extracts with 0.1 *M* phosphate buffer (pH 7.8) were dialyzed thoroughly at 4 C against the 8 buffers of different compositions shown in Table 1 and 2, and then each dialyzed extract was examined for residual η activity. Variance analyses showed that a higher pH than 7.5 and higher salt concentration than 0.1 *M* was critical for stabilizing the η activitity. Another experiment showed that a concentration of 0.07 *M* of phosphate buffer (pH 7.7) was the lowest possible for retaining η activity at 4 C.

The apparently favorable effect of EDTA was confirmed as follows: When heavy metal salts (Mg acetate, Ca acetate, and ferric ammonium citrate) were added to a final concentration of 1 m*M* to freshly prepared cell extracts, a precipitate appeared in all cases but only the supernatant with the ferric iron salt lost η activity completely. Therefore, we investigated methods to remove iron as completely as possible with activated calcium phosphate gel or chelating agent, as described in the Materials and Methods. Precise analyses showed that 0.1 *M* " deferrated " Na (or K) phosphate buffer, pH 7.7 (7.6∼7.8), containing 1 m*M* EDTA, 0.5 m*M* EDDHA and 0.02 *M* Na acetate (coded dPB-EDD), was suitable for preparing *M. tbc* sonic extracts retaining η activity for at least 2 weeks at 0∼4 C after filtration through an EG type membrane filter. (Phosphate buffer dPB-ED is dPB-EDD without EDDHA.)

3. *Conditions for detecting η precipitinogen reproducibly by agar gel diffusion*

In factorial analyses of conditions required for detecting η precipitinogen on immunodiffusion, the following four factors were examined: (i) the nature of the gel (2 kinds of agar and 5 kinds of agarose), (ii) the concentration of gel, (iii) the media for the gel and (iv) the temperature of incubation (4 C, 20 C 25 C and 37 C). Results showed that for immunodiffusion of η precipitinogen in sonic extracts gel prepared by the method described in the Materials and Methods was essential and that incubation of the gel at 4 C overnight and then at 25 C for 1 day was necessary to obtain a sharp distinct η band reproducibly.

4. *Ammonium sulfate fractionation of H37Ra cell extract*

Assuming that other factors contributing to the lability of η precipitinogen were some substances co-extracted from the cells that formed agglutinates with η, we tried to purify η precipitinogen for the stable preparation as reference antigen for long term use. The optimal range of ammonium sulfate saturation for selective precipitation of η precipitinogen was determined as follows. Samples of 2 ml of H37Ra extract, diluted with dPB-ED to give 1.0 mg protein per ml were treated with dSAS (pH 7.7), at 30, 35, 40, 45, 50, 60 and 70% saturation, respectively, (Fig. 2) and left overnight at 4 C. The supernatants obtained by centrifugation at 4 C at 10,000 $\times g$ for 30 min, were then treated with dSAS at 70% saturation. The precipitates were dissolved in 1 ml of dPB-ED, dialyzed overnight and examined for η precipitinogen by immunodiffusion.

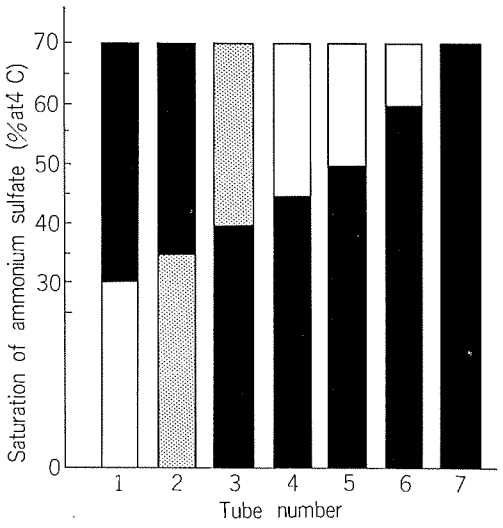

FIGURE 2. Distribution of η activity in ammonium sulfate fractions of H37Ra extracts. Black column, η precipitinogen positive; grey column, trace amount of η precipitinogen; white column, no η precipitinogen activity.

Figure 2 shows that most of η precipitinogen precipitated at a saturation of more than 40%, but not at a saturation of less than 35%.

Therefore H37Ra cell extracts were fractionated twice as follows: First, extracts at 1~2 mg protein/ml were fractionated by precipitation with 40% saturation of the supernatant at 33% saturation of dSAS. Second, the supernatant obtained by treatment with 35% saturation of the first η fraction (1 mg protein/ml solution in dPB-ED) was dialysed in a Visking tube against 42% saturated dSAS at 4 C to give final saturation of 40%.

The partially purified η precipitinogen thus obtained (35–40% dSAS fraction) was stable at 4 C in the form of a precipitate at a saturation of dSAS of more than 40%. But in solution it had to be dialyzed rapidly at 4 C against dPB-ED buffer and purified further within one week to avoid loss of activity.

5. *Gel filtration of the ammonium sulfate fraction (35–40% dSAS fraction) through Sephadex G-200*

Figure 3A shows the elution profile of 280 nm absorbance of the ammonium sulfate fraction (35–40% dSAS fraction) of H37Ra extracts, on Sephadex G-200. η Activity in (Fig. 3) was determined by the micro-modification by Crowle (1958) of Ouchterlony's immunodiffusion.

To obtain a highly purified preparation of η precipitinogen, fractions showing η activity obtained in several experiments were pooled, concentrated by ultrafiltration and rechromatographed.

Figure 3B shows the elution profile on repermeation chromatography of the η precipitinogen fractions (Zone N in Fig. 3A). On rechromatography, the η precipitinogen preparation gave a single protein peak with a slight tail. The η precipitinogen activity almost coincided with the protein peak (Fig. 3B). We used this rechromatographed preparation as purified η precipitinogen in further experiments.

The following experiment was made to determine the exact eluting position of η precipitinogen and the degree of its resolution from other antigens. Fractions (No. 46–61 in Fig. 3A) eluted from the column, including η precipitinogen and its neighbouring fractions, were pooled, concentrated by ultrafiltration in a collodion bag, and subjected to repermeation chromatography through a Sephadex G-200 column (Fig. 4A). The η precipitinogen in each fraction of eluate was arranged quantitatively by rocket-line immunoelectrophoresis using purified η precipitinogen for the intermediate gel (Fig. 4B). The results showed that the η preparation purified by the above method contained several other antigens whose peaks overlapped η fractions (Fig. 4B).

6. *Other methods tested for further purification of η precipitinogen*

Many antigenic substances still remained in the purified η preparation obtained by the

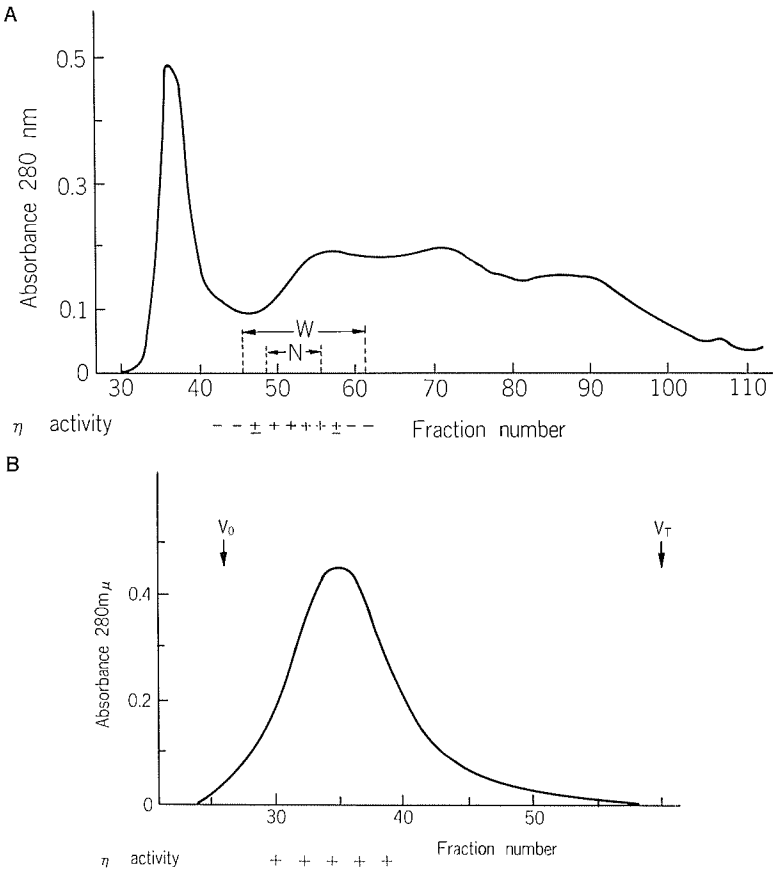

FIGURE 3. Gel permeation chromatography through a " deferrated " Sephadex G-200 column with dPB-ED buffer as eluant at 4 C: of the (A) ammonium sulfate fraction (35–40% saturation) of H37Ra sonic extracts (column, 5 $cm^2 \times 90$ cm; sample, 6.0 ml containing 60 mg protein; fraction size, 4.1 ml; flow rate, 10 ml/h, upward), and (B) fractions corresponding to the proximal two thirds of the peak of η precipitinogen activity, shown as " N " zone in (Fig. 3A), were pooled, concentrated and rechromatographed (column, 1.63 cm^2 $\times 92$ cm; sample, 1.3 ml containing 4.3 mg protein; fraction size, 1.5 ml; flow rate, 3 ml/h). V_O, Void volume: V_T, total volume.

above methods, as shown in Fig. 4 by the limited resolution achieved in these methods. Since even some lots of purified η lost activity, other purification methods were tested under conditions that permitted retention of η activity.

Figure 5 shows the zone-electrophoretic pattern of the ammonium sulfate fraction 35–40% dSAS fraction) of η from strain H37Ra. On zone-electrophoresis, the η precipitinogen moved to the anode. However, the sections containing η precipitinogen also contained other antigens, scarcely any of which could be separated even by gel permeation chromatography.

The anion exchangers tested, DEAE-, TEAE-, AE-, GE- and ECTEOLA-cellulose and DEAE-Sephadex bound η precipitinogen

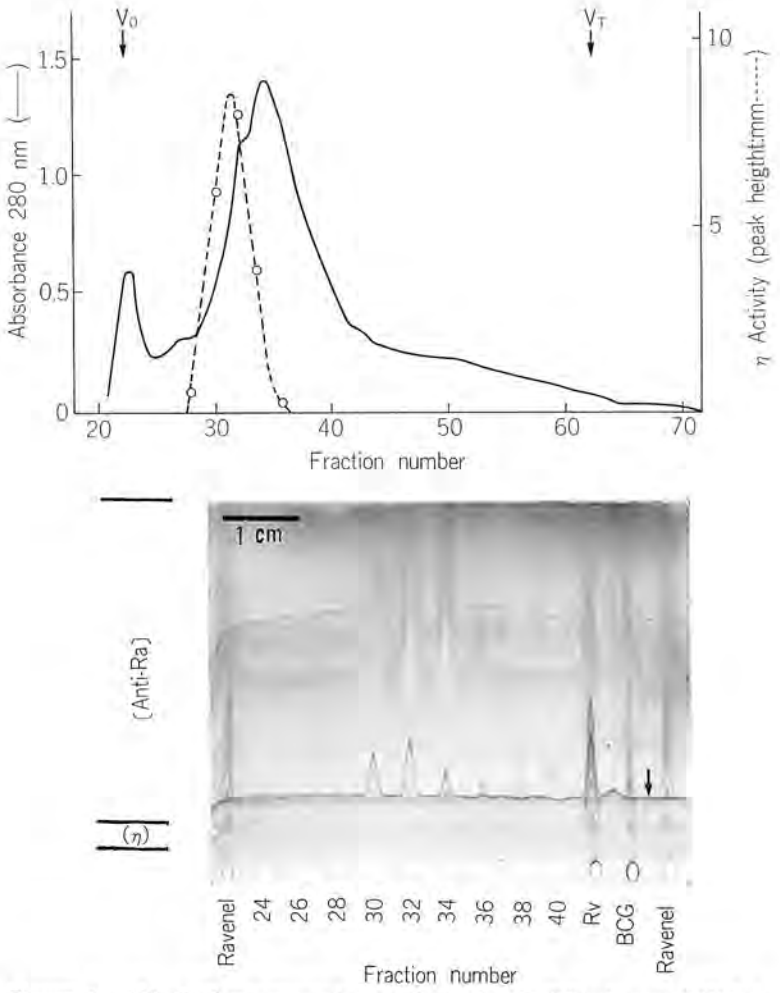

FIGURE 4. (A) Rechromatography on Sephadex G-200 of zone " W " in (Fig. 3A) of the Sephadex fractions (column, 1.52 $cm^2 \times 80.5$ cm; sample, 20 mg protein in 1.3 ml; fraction size, 1.5 ml; flow rate, 2.3 ml/h, upward). V_0, Void volume; V_T, total volume. (B) Rocket-line immunoelectrophoresis of rechromatographed Sephadex fractions (No. 24–40; Fig. 3B) of sonic extracts from H37Ra and from H37Rv (Rv of *M. tuberculosis* and from BCG (BCG) and Ravenel strain (Ravenel) of *M. bovis*. Antibody gel, [Anti-Ra], containing the gammaglobulin fraction of rabbit antiserum against H37Ra extracts. Intermediate gel, (η), containing purified η precipitinogen (20 μg per ml). The bar indicates 1 cm length. The arrow indicates the precipitation line of η precipitinogen by line immunoelectrophoresis.

irreversibly, even at such a high ionic strength as that of 0.5 *M* phosphate buffer (deferrated). Gel-adsorption on hydroxylapatite, and cation exchange with SP-Sephadex also did not separate η precipitinogen from other materials. Moreover on hydrophobic chromatography on Phenyl-Sephacel, η was not released even with 40% ethyleneglycol in 0.1 *M* phosphate buffer.

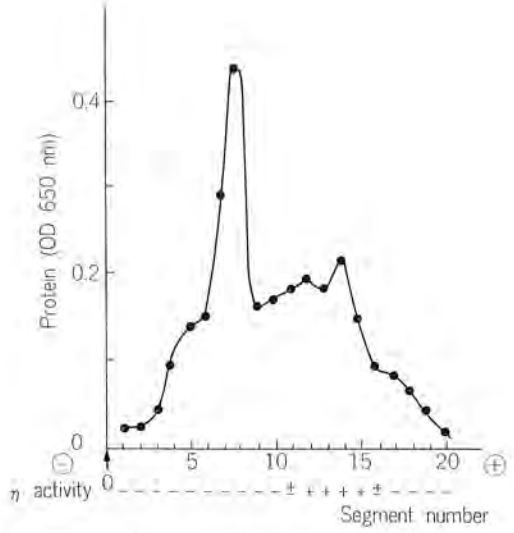

FIGURE 5. Zone-electrophoretic pattern of ammonium sulfate fraction (35–40% saturation) of H37Ra extracts. Protein concentration of separated eluates was determined by the method of Lowry et al. as modified by Hartree and expressed by optical density (OD) at 650 nm.

7. *Characterization of η precipitinogen*

The elution volume (Kav) of η precipitinogen on Sephadex G-200 column chromatography was determined as 0.23 (Fig. 4A), by locating the peak of η precipitinogen activity exactly by rocket-line immunoelectrophoretic assay (Fig. 4B). The molecular weight of η precipitinogen was estimated as 230,000~260,000 daltons from the Kav and elution positions of protein marks as described in the Materials and Methods.

Since η precipitinogen was firmly adsorbed to polyacrylamide gel, conventional polyacrylamide gel electrophoresis was not applicable. In the presence of sodium dodecyl sulfate (SDS), precipitinogen lost its antigenic activity irreversibly. Therefore, although we observed several protein bands on SDS-gel electrophoresis, we could not determine which was that of the η precipitinogen, and so could not determine the molecular weight by this methods.

The purified η precipitinogen preparation

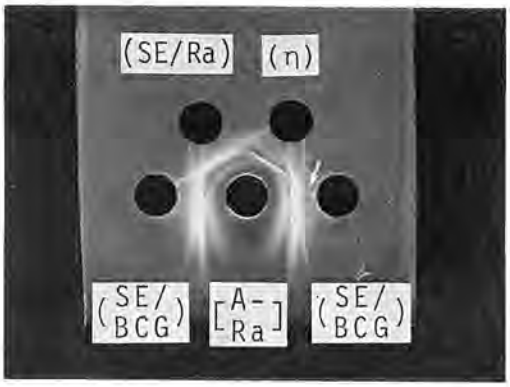

FIGURE 6. Immunodiffusion pattern of purified η precipitinogen against rabbit anti-H37Ra extract serum ([A-Ra]) using BCG extract ((SE/BCG)) as a reference antigen preparation containing no η precipitinogen. ((η)), purified preparation of η precipitinogen (10 μl of antigen solution containing 20 μg protein per ml.).

obtained by a combination of repeated ammonium sulfate fractionation and gel permeation chromatography through a specially pretreated Sephadex G-200 column, formed essentially a single precipitation band against anti-crude H37Ra extract serum (Fig. 6), though it still gave several precipitation arcs by more sensitive methods such as crossed immunoelectrophoresis (Fig. 7A). The arc of η precipitinogen could be identified by crossed-rocket-line immunoelectrophoresis (Fig. 7B). These two methods (Fig. 7A, 7B), also showed that the η precipitinogen had a negative charge. The electrophoretic mobility of η precipitinogen relative to that of the tracking dye (bromophenol blue) was 0.60 (Fig. 7).

In sharp contrast to the η precipitinogen activity in crude sonic extracts, that of the purified preparation was stable: The η activity was not lost on incubation at 40 C for 30 min but was lost on heating at 60 C for 10 min (Fig. 8), or in the frozen state (at −20 C) for several years.

From the results of rocket-line immunoelectrophoresis in Fig. 4B, the purity of the η preparation was estimated as more than 60

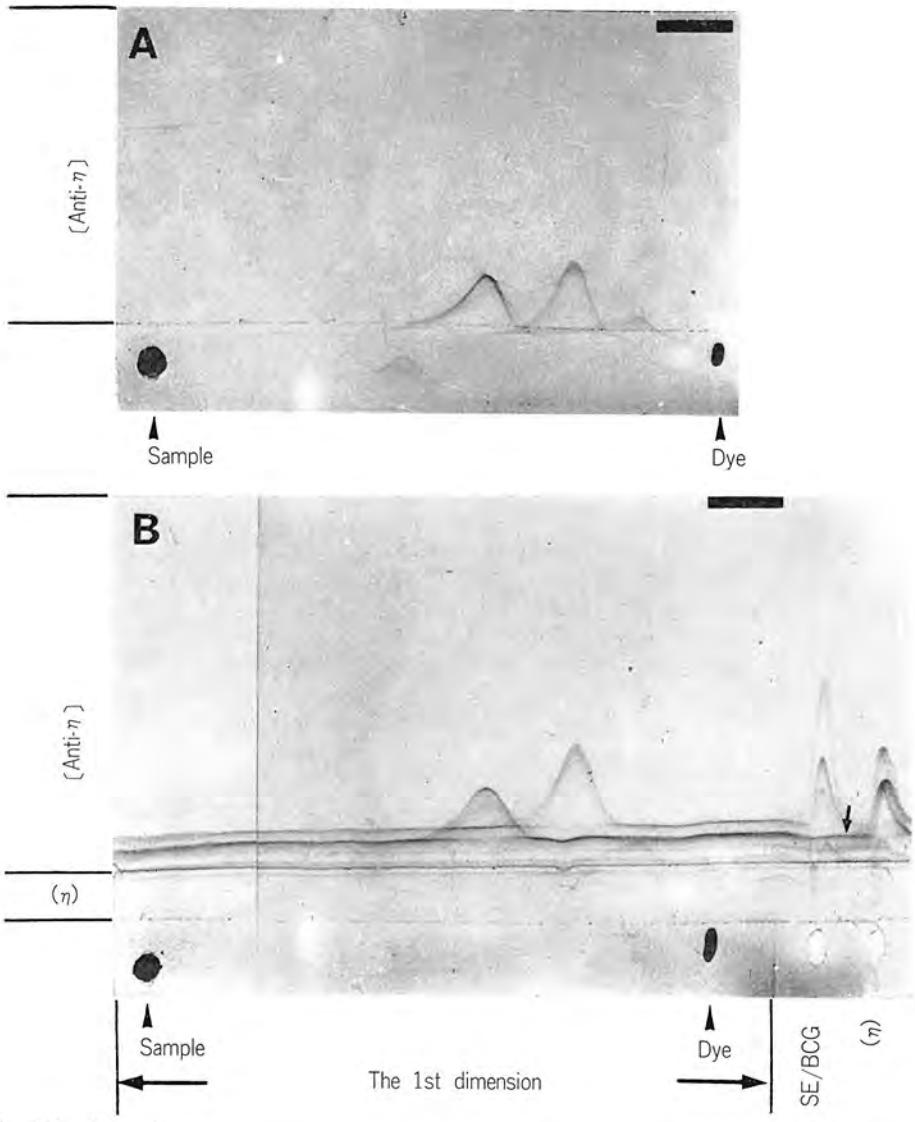

FIGURE 7. (A), Crossed immunoelectrophoresis of purified η precipitinogen against rabbit anti-purified η serum ([Anti-η]) and (B), combination of gel electrophoresis of crossed-line immunoelectrophoresis of the purified preparation of η precipitinogen and of rocket-line immunoelectrophoresis of BCG extracts and the purified η precipitinogen (η). Bars indicate 1 cm length. Dye, bromophenol blue. The arrow indicates the precipitation line of η precipitinogen.

times that of the crude extract, as calculated from the η rocket-heights of fraction No. 32 and H37Rv cell extract and the respective protein concentrations, assuming the η rocket-height above line η is stoichiometrically related to the amount of η introduced into well.

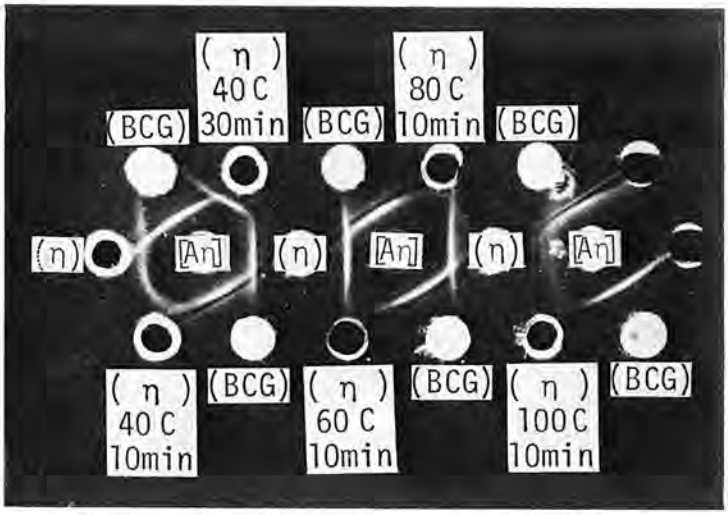

FIGURE 8. Immunodiffusion pattern demonstrating that η precipitinogen in the purified preparation was stable at 40 C for 30 min, but labile on heating at 60 C for 10 min. [Aη], antiserum against purified η precipitinogen; (η), purified preparation of antigen η (100 μg protein per ml); (η 40 C 10 min), (η 40 C 30 min), (η 60 C 10 min), and (η 80 C 10 min), samples of η heated at various temperatures for the time indicated.

DISCUSSION

We immunized many rabbits with various antigen preparations of *M. tbc*, and fortunately obtained a particular antiserum with which we could find a very labile, unique precipitinogen (which we named η). We then purify this precipitinogen in a stable form. The reproducible preparation of cell extracts retaining η activity and of purified, stable η precipitinogen described in this paper, enabled us to prepare reproducibly anti-η antisera. Using these precipitinogen preparations and antisera, we confirmed our initial finding that there is at least one soluble precipitinogen in *M. tbc* strain H37Ra and H37Rv that is undetectable in *M. bovis* strain BCG and Ravenel.

In the early stage of this study, this η precipitinogen was very labile in crude extracts and gave inconsistent results on immunodiffusion. Moreover, production of anti-η antibody in rabbits was very variable, even with a single extract containing the precipitinogen. This reflects the fact that antigens that are detectable using antiserum, a product of a living being in response to antigens, are only some of various *elements* involved in the *system* for preparing and detecting antigens. These *elements* in the antigen-detecting *system* for mycobacteria have never been systematically analyzed. The *system* that we developed in this study by focusing on one unique, single precipitinogen η is composed of the following *elements*: (Italic letters indicate newly investigated or specifically selected methods indispensable in the detecting system) (A), Preparation of sonic extracts for precipitinogen; (A1) *M. tbc* H37Ra and H37Rv and *M. bovis* BCG and Ravenel, (A2) culture methods, media and culture age; *cells harvested after 2 weeks of shaking culture and growth on glycerin-broth-potato*, (A3) collection of pathogenic cells by centrifugation and *storage at −20 C or −70 C*, (A4) extraction of antigens from cells; prevention of biohazard from aerosol and cooling during sonication of cells; *complete im-*

mersion of the vessels and tip of the sonicator in an ice-water bath during sonication, (A5) fractionation of the extracts; centrifugation (final 100,000 $\times g$ for 2 h) and *filtration through a membrane filter of cellulose acetate or regenerated cellulose type* for removing remaining cells, (A6) storage of the extracts *at 0 C~4 C in the unfrozen state*, (A7) preparation of buffer for extracting the precipitinogen; *deferration of phosphate buffer* and *its adjustment to above pH 7.6 and a concentration of more than 0.07 M and addition of chelating agents in acetate*, (B), Detection of the η precipitinogen band; (B8) *preparation of deferrated agar*, (B9) preparation of antisera; Freund's complete adjuvant using a homologous strain, (B10) temperature for incubation of the reaction gel; *initial incubation at 4 C at least overnight*, (B11) fused-cross or rocket-line immunoelectrophoresis; *preparation of agar and electrode buffer*, and *stable reference antigen of* η; (C) Purification of η precipitinogen for reference antigen; (C12) purification by salting-out with ammonium sulfate; *deferration of saturated ammonium sulfate solution* and *fractionation at 35–40% saturation*, (C13) gel permeation chromatography; *method of washing matrices.*

The antigenic composition of preparations from cells and culture filtrates have been compared by Seibert (1950), Castelnuovo (1964), Daniel et al. (1975), and Janicki et al. (1976), who conclude that there was no remarkable difference in the antigenic composition of the two kinds of preparation. In the case of η, we observed difference in the amounts of η precipitinogen in the two kinds of preparations depending on the medium employed. We used cells in shaking culture for extraction of η precipitinogen, since cells grown by surface culture on Sauton's medium did not show any η activity but had many other antigenic activities, whereas culture filtrates of both shaking and surface cultures showed η activity when highly concentrated (about 200-fold concentration in a collodion bag): The reference η band curved slightly to the antigen wells of test materials. This indicates that most of the η precipitinogen is localized in or on the organisms, and that suitable media and methods of culture should be selected for preparing η precipitinogen.

There have been many reports on the effect of cultures on their antigenic composition (Magnusson et al., 1964; Dardas et al., 1968; Turcott, 1969; Janicki et al, 1976). No remarkable influence was observed. But η activity in extracts of cells from old cultures was lower than that from cells of young cultures and was lost more rapidly (within a few days even if prepared under optimal conditions). Turcott (1972) pointed out the influence of the culture age on the chemical composition of the culture. His results suggested that the early phase of culture was favorable for preparing protein antigens as certain polysaccharides or lipopolysaccharidies increased in old cultures. So the low η activity and its rapid loss in extracts from old cultures may be due to precipitation of η by some co-agglutinating factors that accumulate in old cultures.

The selective permeation of η precipitinogen through cellulose acetate and regenerated cellulose types of membrane filters suggests the presence of a hydrophobic area in η precipitinogen in phosphate buffer, since these two types of membrane are more hydrophilic than the other types of membrane filters tested. But addition of a detergent, Tween 80, ND40 or SDS, rapidly inactivated η in sonic extracts, possibly due to impurities in the detergents and/or conformational change of the η molecule.

The high pH of the buffer required may act (1) in solubilizing η, which is a large, partially hydrophobic protein molecule, and (2) in accelerating heavy metal chelation by reagents. The amount of precipitate appearing in sonic extracts immediately after lowering the pH to below 7.5 was much larger than the estimated amount of η. Therefore, the most likely aggregating factors were materials co-extracted with η precipitinogen and heavy metals. Polycations, low pH and low ionic strength may have unfavorable effects on ac-

tivity by causing formation of aggregates including η precipitinogen.

No apparent difference was observed between the effects of Tris and phosphate buffer on η activity in the experiments described in 1 and 2. However, when Tris buffer was used for extraction, the η activity of the extract was less than when phosphate buffer was used and the activity was lost within a few days. Possible reasons why phosphate buffer is more suitable than Tris buffer are as follows: (1) Phosphate has a larger buffering effect than Tris buffer and, unlike Tris, its buffering effect increases with decrease in pH below pH 7.8. This character of phosphate buffer may be favorable in preventing decrease in pH of sonic extracts, which generally occures during disintegration of the cells, because η precipitinogen is unstable below pH 7.5. (2) Phosphate ion may act as a polyanion against acidic protein, inhibiting colloidal hetero-coagglutination. (3) η Precipitinogen may be an enzyme of phosphate metabolism. (4) A high concentration of phosphate may prevent η from inactivating with heavy metals. (5) Deferration of Tris buffer was less complete than that of phosphate buffer.

Loss of η activity by SH reagents may be due to (1) lowering of pH of the cell extracts, (2) activation of SH-protease, (3) masking or altering η antigenic sites, or conformational changes of η protein by regional opening of S-S bonds, (4) conformational changes by actions on protein-Cys-S, which might be present in the iron binding site of η, as iron-binding protein, as in ferredoxin.

The effect of iron on η activity may be specific or possibly be that of a polycation causing co-agglutination. Mycobactin T, if related to η, may serve to form η antigenic sites by binding to a component of η and iron may liberate mycobactin from η precipitinogen, resulting in loss of η activity.

Though η antigen can now be prepared reproducibly from cells by the system described above (A group), the η activity in crude sonic extracts can not be retained for more than 2 weeks even in the unfrozen state (at 0~4 C).

With regard to technical factors affecting the appearance of precipitin reactions in agar, Janicki et al. (1973) reported differences in patterns on Grabar's type immunoelectrophoresis, but they did not test differences in various lots of agar product and in its contaminants. Their results may be explained by differences in electrosmosis mainly due to sulfonic radicals of agar.

As preservatives, merthiolate (0.01%) and phenol (0.1%) were unfavorable for η activity. Organic mercury in the former and the hydrophobic character of the latter may have unfavorable effects. Azide, which has metal chelating activity, had a less unfavorable effect on η activity.

The incubation temperature of reaction in agar (4 C) required in the η detecting system resulted in the appearance not only of a reproducible η band, but also of other precipitinogen bands that were well separated from each other.

At an early stage of this study, Ouchterlony's double immunodiffusion method was used for identifying precipitation bands. But, when a stable, purified preparation of η precipitinogen was obtained, rocket-line immunoelectrophoresis could be applied. For high resolution and simultaneous identification of multiple antigens of thermolabile antigens, Chaparas's modification (1978a) of fused-rocket immunoelectrophoresis is very useful. Rocket-line immunoelectrophoresis (Krøll, 1973) can also be used to determine the amounts of individual antigens, if the antigen under investigation is relatively thermostable. The latter method could be used with the purified preparation of η precipitinogen described above, which is stable at 40 C for 30 min. If a method can be devised for application of antigens to intermediate gel at lower temperature (e.g., 4 C), rocket-line electrophoresis will be more useful in quantitative studies of the antigen compositions of various preparations.

The zone (35–40%) in which η precipitinogen was precipitated by dSAS was similar to that

of gamma-globulin. Like the latter, η precipitinogen requires a high pH and high concentration of salt. We found that gamma-globulin of rabbit antisera was precipitated at 30–40% saturation of dSAS at 0 C after dilution with phosphate buffer (the Materials and Methods) though gamma-globulin is generally precipitated at 30% saturation of ammonium sulfate at room temperature. Thus, η precipitinogen, molecular weight 230,000~260,000 daltons, behaves like gamma-globulin.

Among the numerous reports on mycobacterial antigens, that of Youmans and Youmans (1964) itemerized similar difficulties in preparing immunogenic factors to those described above. They pointed out that the following factors influence the lability of their preparation; culture age, temperature for preparing the antigen, detergent, hydrogen-ion concentration, storage by lyophilization or freezing, dialysis against distilled water, and metal ions, though they could not find suitable conditions. The conditions that inactivated their immunogenic factors resemble inactivating the η precipitinogen. However, it is unlikely that our preparation of η precipitinogen contained their immunogenic factors, since their particulate immunogenic factors, if present, were removed from sonicated preparation by ultracentrifugation. On the other hand, their immunogenic factors may have contained η precipitinogen in a form bound to their particulate fraction, since under the conditions they described η precipitinogen, if present in the crude extracts, would have been precipitated in the particulate fraction.

Ouchterlony et al. (1968) emphasized that even if a crude preparation containing several antigens is used as test material for classification of mycobacteria, the antigenic composition will be revealed by the precipitation pattern, provided that a proper polyvalent serum is employed. As we showed above, the procedures for preparation of precipitinogens that are native and stable enough for producing a proper polyvalent antiserum and are soluble or diffusible in gel for the precipitation reaction, are the most important for reproducible analysis of antigenic composition. Our system using deferrated-buffer of high ionic strength for cell extraction is in fact very different from, for example, the US-Japan reference system (Janicki at al., 1971). The latter system differs in the following points (the codes of items described above are shown for comparison); Janicki et al. used culture filtrates (A2), harvested from 8 week cultures ages (A2), sterilization with a cellulose nitrate membrane filter (A5), dialysis against distilled water (A7), lyophilization (A6), 0.9% sodium chloride with no buffering action to solubilize the lyophilized antigen (A7), and removal of insoluble material by refiltration through a membrane filter (A5). Owing to the electroviscous effect of the solution of low ionic strength employed in the reference system, most large molecular weight antigens would be rapidly inactivated during the preparation. Actually, Minden and Farr, and Knicker and Lanigan (Mycobacterial Antigen Workshop, 1972) suggested that antibodies against certain antigens were not present in US-Japan reference antisera. Therefore, it is unlikely that η precipitinogen and its antibody are present, and the η precipitinogen band may have been detected in the US-Japan reference system, though final conclusion must await further study using η precipitinogen and its antibody. The system of Lind (1960) could not demontrate any difference between *M. tbc* and *M. bovis* BCG. The antiserum of Roberts et al. (1972) may contain anti-η antibody, because as the antigen preparation of *M. tbc* for immunization, they used the "15,000 $\times g$", which would contain η precipitinogen in an agglutinated form under their conditions for preparing cell extracts.

Though it is not certain whether our system for preparing and detecting η covers all antigens that have been detected by other systems, still this system may be better for detecting protein antigens than the conventional US-Japan reference system. A system developed for certain antigens may sacrifice some

other antigen activities. Therefore, several systems, including the phenol extraction described by Wayne (1971) for non-protein antigens, are required for serological comparison of mycobacterial species. A set of stable, well characterized reference antigens will be useful in developing a simpler method for taxonomical identification of mycobacteria than the conventional methods, which are intended to have as many antigens as possible in one reference system.

Using the system described in this report, we are now examining whether η precipitinogen is species-specific for *M. tbc* by testing various species of mycobacteria including *M. bovis*. The results will be published elsewhere.

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